

Amendments to the Specification

Please change the title to the following:

ADIPONECTIN EXPRESSION-INDUCING AGENTS AND USES THEREOF

At page 1, after the title, please insert:

RELATED APPLICATIONS

This application is a National Phase Filing of International Patent Application No. PCT/JP2005/006357, filed March 31, 2005, which claims priority to U.S. Provisional Patent Application No. 60/557,708, filed March 31, 2004, the disclosures of each of which are hereby incorporated by reference.

Please replace the paragraph beginning at page 4, line 1 with the following:

Electrophoretic mobility shift assay (EMSA) showed that a 32-bp fragment of position -188 to position -157 from the transcription start site of the adiponectin gene binds to proteins in nuclear extracts prepared separately from adipocytes and adipose tissue. Substances that decrease the electrophoretic mobility of the 32-bp element were found mostly in the nuclear extracts prepared from small adipocytes, as compared with those prepared from large adipocytes. Similarly, more of such substances were found in the nuclear extracts prepared from adipose tissues of lean mice than those from obese mice. A nuclear factor that binds to the 32-bp fragment was identified using the yeast one-hybrid screening method. Six positive factors obtained from the one-hybrid screening were further analyzed by EMSA and ~~chromosome~~ chromatin immunoprecipitation assay, and Kruppel-like factor 9 (hereinafter abbreviated as "KLF9") was identified. KLF9 binds to the above-mentioned element, and the binding level correlates with the *in vitro* and *in vivo* KLF9 expression levels. In addition, cotransfection experiments showed that transient overexpression of KLF9 enhances adiponectin promoter activity specifically and dose-dependently. *In vitro* suppression of KLF9 expression by siRNA and KLF9 overexpression as well as *in vivo* KLF9 knockout cause changes in the endogenous

adiponectin mRNA level. This confirmed that transcriptional regulation of adiponectin is carried out by KLF9. These results suggest that if supplemented to swollen hypertrophic adipocytes, KLF9 can be used as a preventive and/or therapeutic agent for obesity or obesity-related diseases such as metabolic diseases including insulin resistance and type II diabetes, and cardiovascular diseases. These results also indicate that KLF9 is important as a target for drug discovery against the above-mentioned diseases. The present invention is based on these findings and specifically relates to:

Please replace the paragraph beginning at page 6, line 6 with the following:

Fig. 2 shows results of measuring the adiponectin promoter activity in 3T3L1 cells during adipocyte differentiation and hypertrophy. Each of the graphs shows results of determining: (a) adiponectin promoter activity in 3T3L1 cells during adipocyte differentiation and hypertrophy; (b) adiponectin promoter activity in 3T3L1 cells incubated with the indicated concentrations of $\text{TNF}\alpha$ 10 days after the induction of differentiation; and (c) adiponectin promoter activity in $\text{TNF}\alpha$ (3 ng/mL)-treated or untreated 3T3L1 cells incubated with an anti- $\text{TNF}\alpha$ antibody 10 or 19 days after induction of differentiation. The (-1367/+35) of the adiponectin promoter-luciferase gene (Luc) expression vector was transiently transfected into 3T3L1 cells. The results in (b) are shown as relative values taking the activity without $\text{TNF}\alpha$ addition as 100%. Each of the bars in (b) shows the mean \pm SE (n = 5 to 7) (*: $P < 0.05$, **: $P < 0.01$; relative to untreated cells). The adiponectin promoter activity in hypertrophic adipocytes is decreased because of the existence of non- $\text{TNF}\alpha$ signal transduction pathways.

Please replace the three paragraphs beginning at page 6, line 36 with the following:

Fig. 4 presents photographs showing the results of analyzing factors that bind to the adiponectin promoter region in the nuclear extract of 3T3L1 adipocytes (day 10). Panels a to d, and f show results of the EMSA analysis. The photographs show (a) results of an

EMSA analysis when the nuclear extracts from 3T3L1 adipocytes (day 10) or 293T cells, and radiolabeled NF- κ B consensus sequence (p65 site) were incubated in the presence or absence of an antibody that specifically recognizes KLF9 or NF- κ Bp65; (b) (right panel) results of incubating the nuclear extract of 3T3L1 adipocytes (two lanes on the left: day 10, two lanes on the right: day 19) with a labeled 32-bp oligonucleotide probe of the adiponectin promoter region (-188/-157) in the presence or absence of an anti-KLF3 antibody, (left panel) results of an EMSA analysis when the nuclear extract from 293T cells and KLF oligo (KLF consensus sequence) were incubated in the presence or absence of an anti-KLF3 antibody; (c) results of reacting the nuclear extract from 3T3L1 adipocytes (day 10) with the KLF9 consensus sequence (BTE) in the presence or absence of an anti-KLF9 antibody; (d) results of reacting WAT from lean control mice C57BL6 (B6) or from obese mouse model ob/ob mice, with the 32-bp fragment and an anti-KLF9 antibody; and (f) results of incubating with the 32-bp fragment, purified FLAG-tag KLF9, or WAT from B6 or from obese mouse model ob/ob mice. The arrows in the figures indicate a supershifted specific complex. (e) A photograph showing results of the ~~chromosome~~ chromatin immunoprecipitation assay with KLF9 bound to an endogenous adiponectin promoter in 3T3L1 adipocytes (day 10). The data shows representative results obtained from a series of three independent experiments. EMSA analyses showed that the 32-bp binding complex contains KLF9 *in vitro* and *in vivo*.

Fig. 5 presents photographs and diagrams showing results of analyzing the expression levels of KLF3 and KLF9 in adipocytes. (a and b) Photographs of Northern blots that analyze the expression levels of KLF3 mRNA (a, top) or KLF9 mRNA (a, lower) in 3T3L1 adipocytes, in which an indicated number of days had passed since differentiation is induced, and in WAT obtained from lean control mice C57BL6 or from obese mouse model ob/ob mice (~~a to e~~) (a to b); graphs that quantify the band intensity; and a photograph showing results of analyzing the expression level of mKLF9 protein (b) by Western blotting. The arrow in (b) indicates KLF9. Each of the bars in the graphs shown in (a) represents the mean \pm SE (n = 3 to 5). KLF9 expression increased during adipocyte differentiation, but decreased during adipocyte hypertrophy.

Fig. 6 presents diagrams and a photograph showing results of analyzing the effect of KLF9 overexpression on adiponectin expression. (a) Results of introducing into 3T3L1 adipocytes (day 19) a vector expressing [[KLF]] KLF9 (KLF9/pcDNA3.1) and a vector (“1367bp-Luc”) equipped with a reporter (luciferase) gene downstream of the adiponectin promoter (“-1367/+35”), and analyzing the effect of KLF9 overexpression. The results are shown as relative activities taking “Mock” (only pcDNA3.1) as 1. PDGF is a positive control that is equipped with a KLF recognition sequence and induces the KLF family expression. (b) Results of measuring the expression level of KLF9 mRNA in 3T3L1 adipocytes (day 19) by Taq-man PCR, when the cells were introduced with KLF9/pcDNA3.1 by lipofection. 1/3000 and 1/1000 indicate the dilution ratios when vector introduction was performed by lipofection, and “0” indicates no introduction. (c) The rate of increase in the reporter gene expression, when 3T3L1 adipocytes (day 19) carrying PDGF-tk-luc or 32 bp-tk-luc as a reporter are introduced with mKLF5/pcDNA3.1 or mKLF9/pcDNA3.1, is indicated as an activity relative to the case without introduction. (d) Results of measuring the expression level of KLF9 when the KLF9 gene is stably introduced into 3T3L1 adipocytes (day 19) using retroviral vectors. The vertical axis “mKLF9/36B4” refers to KLF9 mRNA expression ratio corrected using the 36B4 mRNA expression level. (f) Results of measuring the expression level of adiponectin when the KLF9 gene was stably introduced into 3T3L1 adipocytes (day 19) using retroviral vectors. (e) A photograph showing the result of preparing a nuclear protein extract from 3T3L1 adipocytes (day 19) introduced with KLF9 using a retrovirus, and subjecting it to EMSA analysis using the adiponectin promoter (-188/-157) sequence as a ³²P-labeled probe. Each of the bars in the figure shows mean ± SE (n = 5 to 7). KLF9 increased activities of the enhancer and adiponectin promoter, amount of the 32-bp binding protein, and expression of adiponectin.

Please replace the paragraph beginning at page 8, line 17 with the following:

Fig. 7 presents a photograph and a diagram showing results of analyzing the effect of KLF9 knockdown by siRNA on adiponectin expression. (a) KLF3, KLF9, and adiponectin mRNA levels 72 hours ~~and 96 hours~~ after introducing a KLF9 siRNA into 3T3L1 adipocytes (day 10). Each of the results was shown as a relative ratio by making the activity without siRNA introduction as 100%. (b) A photograph showing results of preparing a nuclear protein extract from cells 72 hours or 96 hours after introducing siRNA into 3T3L1 adipocytes (day 10), and subjecting this to EMSA analysis using the adiponectin promoter (-188/-157) sequence as a ³²P-labeled probe. Suppression of KLF9 expression by siRNA decreased the amount of the 32-bp binding protein and adiponectin expression *in vitro*.

Please replace the paragraph beginning at page 8, line 31 with the following:

Fig. 9 shows the mechanism by which adipocyte hypertrophy regulates KLF9 expression in adipocytes. (a) The mRNA expression level of thyroid hormone receptor α (TR α) in 3T3L1 adipocytes (day 10 or day ~~[[17]]~~19). (b) TR α mRNA expression levels in lean C57BL mice and obese ob/ob mice. (c) KLF9 mRNA expression levels after T3 treatment at concentrations indicated in the figure are shown as ratios relative to the 36B4 mRNA expression level. (d and e) The mRNA expression levels of KLF9 or adiponectin in 3T3L1 adipocytes (days 6 and 13), and in 3T3L1 adipocytes (day 19) treated with N-acetyl cysteine (NAC) (20 mM) which is an antioxidant, SP 600125 which is a JNK inhibitor, or NAC(SP). In the figure, NAC(SP) refers to the group treated with NAC and SP600125.

Please replace the paragraph beginning at page 10, line 23 with the following:

In addition to the above-mentioned methods, examples of methods for obtaining proteins that are functionally equivalent to KLF9 include methods that modify the DNA of SEQ ID NO: 1 and then synthesize proteins based on the modified DNAs. Proteins

obtained by artificially modifying mouse KLF9 (SEQ ID NO: 2), and proteins encoded by polynucleotides isolated using the above-mentioned hybridization techniques and such are usually highly homologous to human KLF9 (~~SEQ ID NO: 2~~) at the amino acid level.

“Highly homologous” refers to sequence identity of at least 30% or more, preferably 50% or more, and more preferably 80% or more (for example, 95% or more). Nucleotide sequence identity and amino acid sequence identity can be determined using Internet homology search websites [homology searches, such as FASTA, BLAST, PSI-BLAST, and SSEARCH can be used through the DNA Data Bank of Japan (DDBJ) [for example, the homology search (Search and Analysis) page on DDBJ website;

~~<http://www.ddbj.nig.ac.jp/E-mail/homology-j.html>~~

<http://www.ddbj.nig.ac.jp/search/Welcome-j.html>]. BLAST searches can be performed with National Center for Biotechnology Information (NCBI) (for example, the BLAST page on the NCBI website; <http://www.ncbi.nlm.nih.gov/BLAST/>; Altschul, S.F. *et al.*, J. Mol. Biol., 1990, 215(3):403-10; Altschul, S.F. & Gish, W., Meth. Enzymol., 1996, 266: 460-480; Altschul, S.F. *et al.*, Nucleic Acids Res., 1997, 25:3389-3402)].

Please replace the paragraph beginning at page 14, line 16 with the following:

“A KLF9-encoding DNA” is the same as the KLF9-encoding DNA mentioned above in the description of adiponectin expression-inducing agents, and the scope of the meaning of this phrase is also the same. Specifically, an example of a KLF9-encoding DNA is a DNA comprising the nucleotide sequence of SEQ ID NO: 1. Additional examples include DNAs that hybridize under stringent conditions with the nucleotide sequence of SEQ ID NO: 1, so long as the adiponectin expression-inducing activity is maintained. KLF9-encoding DNAs may be endogenous DNAs in the cells or exogenously introduced ~~[[cells]]~~DNAs. Such KLF9-encoding DNAs are preferably equipped with an intrinsic regulatory region of the KLF9 gene in their upstream region.

Please replace the paragraph beginning at page 24, line 2 with the following:

~~Chromosome~~ Chromatin immunoprecipitation assay was performed to further confirm these findings. KLF9 was found to actually bind to the endogenous adiponectin promoter region comprising the 32-bp site (Fig. 4e). Furthermore, purified KLF9 also showed nearly the same inhibition of the 32-bp fragment as that by a nuclear extract prepared from adipocytes or adipose tissue (Fig. 4f).

Please replace the paragraph beginning at page 25, line 12 with the following:

Next, to study the functional relationship between adiponectin expression and KLF9 *in vivo*, the phenotypes of KLF9 knockout mice were analyzed (~~Fig-8a~~) (Fig. 8) (Morita, M. *et al.*, Mol. Cell. Biol. 23, 2489-2500 (2003)). Interestingly, the 32-bp binding protein was not detected in the nuclear extract derived from KLF9 knockout mice “WAT” (~~Fig-8b~~) (Fig. 8). Importantly, despite that the weight of KLF9 knockout mice was lower than that of the control wildtype littermates, the plasma adiponectin level in KLF9 knockout mice was lower than that of the control wildtype littermates (~~Fig-e~~) (Fig. 8). In contrast, no difference in the plasma adiponectin level was observed between KLF3 knockout mice and their control wildtype littermates (data not shown). These data suggested that KLF9 plays an important role in the regulation of the adiponectin level *in vivo*.

Please replace the paragraph beginning at page 26, line 10 with the following:

To further confirm the above-mentioned hypothesis, the change in oxidative stress during adipocyte hypertrophy was measured. Genomic DNAs extracted from 3T3L1 cells (day 10 and day ~~[[18]]19~~) were degraded, and the amount of 8-OHdG (oxidized form of dG) in the degradation products of genomic DNA was measured by ELISA using a specific antibody. The amount of 8-OHdG increased along with differentiation (Fig. 10). The change of antioxidative activity that accompanies adipocyte hypertrophy was

examined to analyze the cause of the hypertrophy-accompanying increase in 8-OHdG. Antioxidative activity was detected as the activity of metmyoglobin to prevent formation of [[ABIT[[ABTS radicals in 3T3L1 cell lysates (day 10 and day [[18]]19). Antioxidative activity was shown to decrease following adipocyte hypertrophy (Fig. 11).